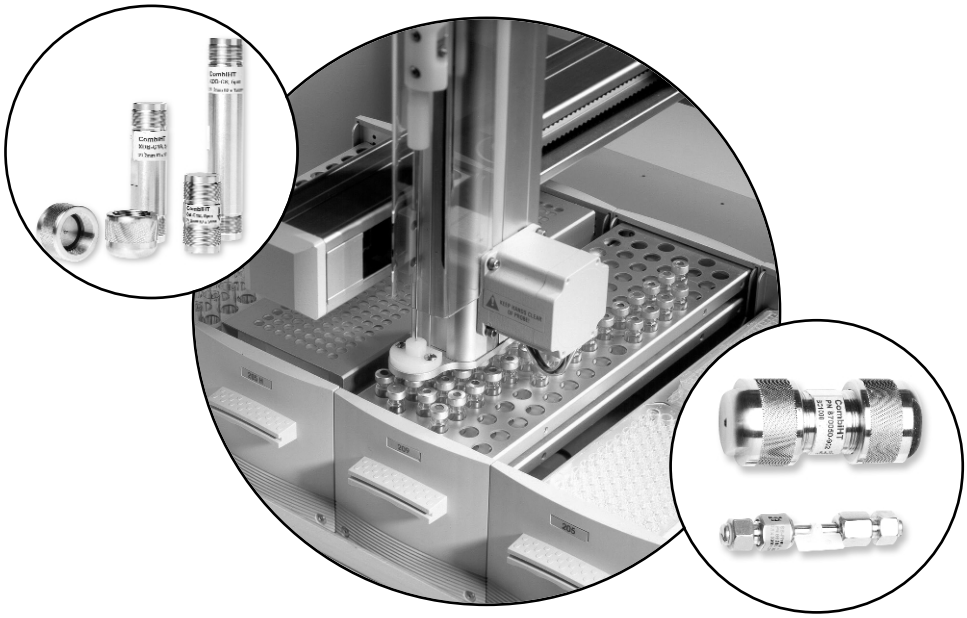
Semi-preparative purification of synthesis products with the Agilent high-throughput analysis system

Application Note

Udo Huber



Abstract

Preparative and semi-preparative HPLC is used for isolation and purification of compounds in the chemical and pharmaceutical industry as well as in biotechnology and biology. The goal is to get a high yield of a product with a certain purity. Therefore, it is necessary to separate discrete compounds from a mixture by fractionation. This can be done easily using the Agilent 220 micro plate sampler with fraction-collection capability together with the Agilent 1100 Series LC system. In this application note we demonstrate the capabilities of the system in a model scenario—the semi-preparative purification of a crude synthesis product.

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Introduction

Semi-preparative HPLC is a technique to isolate and purify compounds from a mixture. The goal is to get the single compounds at a certain purity level.

The difference between analytical and preparative HPLC is the amount of sample applied to the column. In analytical HPLC the applied sample amount is very small compared to the amount of stationary phase in the column (less than 1:10000). Therefore very good separations can be achieved. To purify higher amounts of sample in a single run the loadability of a column has to be increased. This can be done either by concentration or volume overloading, depending on the application.

Since the preparative separation consumes a lot of sample and eluent the development of the method is usually not done on the preparative system. The optimization of the method can be done on an analytical instrument and the optimized method is then scaled up to preparative scale.

The optimization process and the final separation can be done in five steps:

1. Evaluation of the optimum separation conditions on an analytical column.
2. Determination of the adsorption isotherm.
3. Systematic loading and overloading of the analytical column.
4. Scale-up to a semi-preparative column.
5. Separation of the crude product sample using the Agilent 220 micro plate sampler.

In this application note we describe a semi-preparative model scenario—the separation of a compound from a side product. In the crude product the purity of the desired compound is about 80 %, the amount of the side product is about 20 %. The goal is to achieve an overall purity of the product of more than 95 %, containing less than 2 % of the side product. Furthermore about 2 mg of the crude product should be separated within one run.

Equipment

The system included an Agilent 1100 Series vacuum degasser, an Agilent 1100 Series quaternary pump, an Agilent 1100 Series thermostatted column compartment, an Agilent 1100 Series diode array detector and an Agilent 220 micro plate sampler with the fraction collection accessory kit.
The system was controlled using the Agilent ChemStation (version A.07.01) and the micro plate sampling software (version A.03.01).

Results and Discussion

Analytical Method

The first step was to develop an analytical method. The gradient and the eluent were selected so that the selectivity α for the compounds to be separated is between 1.5 and 3. The modification of the method is displayed in figure 1.

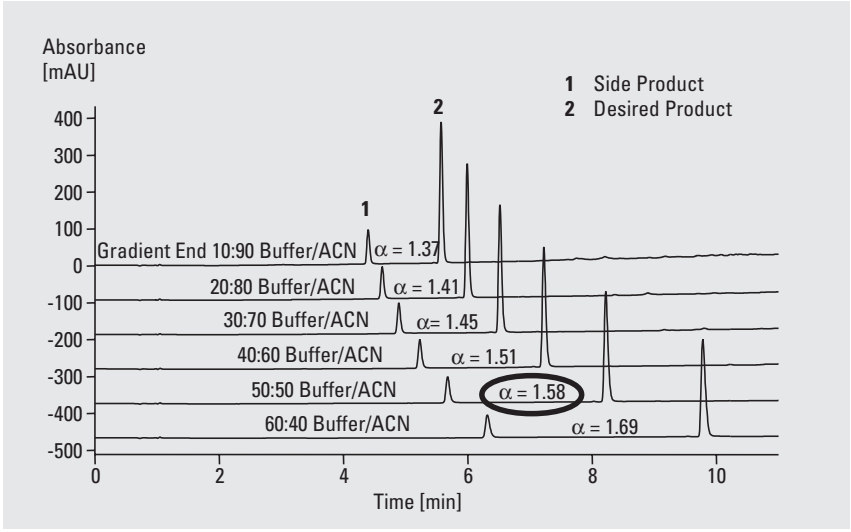

Figure 1
Development of the analytical method

Mobile Phases	A=0.025M KH_2PO_4 in water (pH=3), B = ACN
Gradient:	0 % B to 50 % B in 10 min 50 % B to 0 % B in 1 min
Flow	1 ml/min
Stop time:	11 min
Post time	5 min
Column:	Zorbax SB-C18, 4.6 x 75 mm, 5 µm
Column temp.:	40 °C
UV detector:	DAD 204 nm/16 (reference 360 nm/100)

Determination of the adsorption isotherm

When small sample amounts are applied to the column the capacity factor and the peakwidth at half height are constant (see figure 2, zone A). This is the usual working area of analytical HPLC. The peak shape in the chromatogram is symmetrical and is very similar to a Gaussian curve. With increasing sample amount applied to the column the linear range of the adsorption isotherm is left, which is indicated by changing capacity factors and unsymmetrical peak shapes (figure 2, zone P). This zone is the non-linear part of the adsorption isotherm, which is the working area of preparative HPLC. In this example the non-linear part of the adsorption isotherm, which is the working area for preparative HPLC, begins between 10^{-5}–10^{-6} g(sample)/g(sorbens).

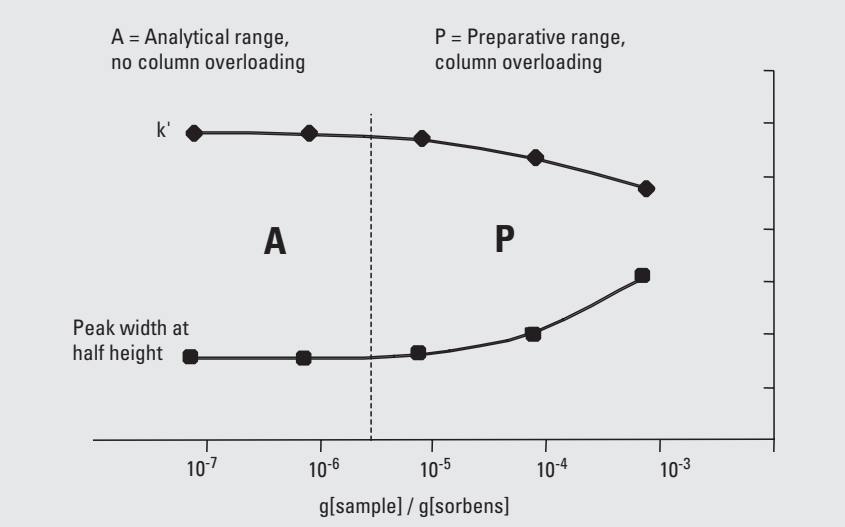

Figure 2
Adsorption isotherm

Loading and overloading of the analytical column

Systematical loading and overloading of the analytical column moves the analysis from the analytical to the preparative zone in the adsorption isotherm diagram. Overloading can be done in two ways: volume overloading and concentration overloading. Which procedure to select depends on the application, specifically on the solubility of the crude product in the eluent. Concentration overloading can only be done effectively if the solubility of the sample is higher than 20 mg per ml eluent. Otherwise volume overloading or a combination of both overloading procedures has to be done. The results for overloading the analytical column are shown in figures 3a and 3b.

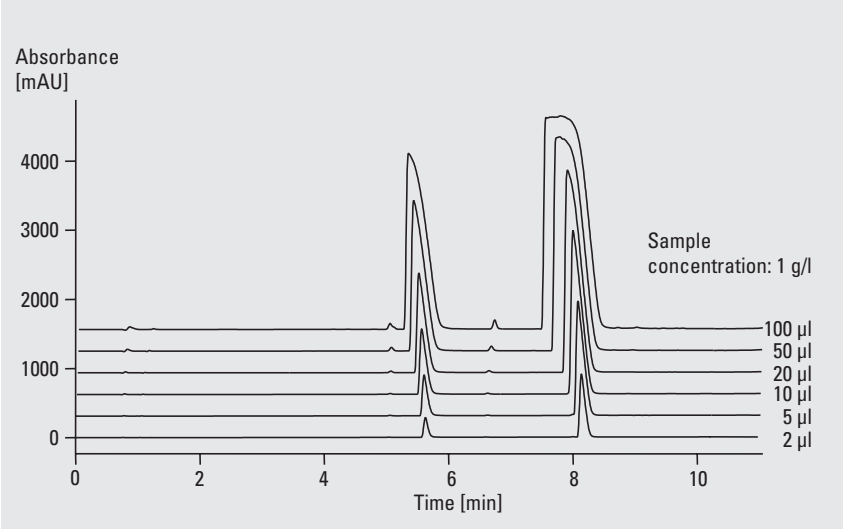

Figure 3a
Volume overloading

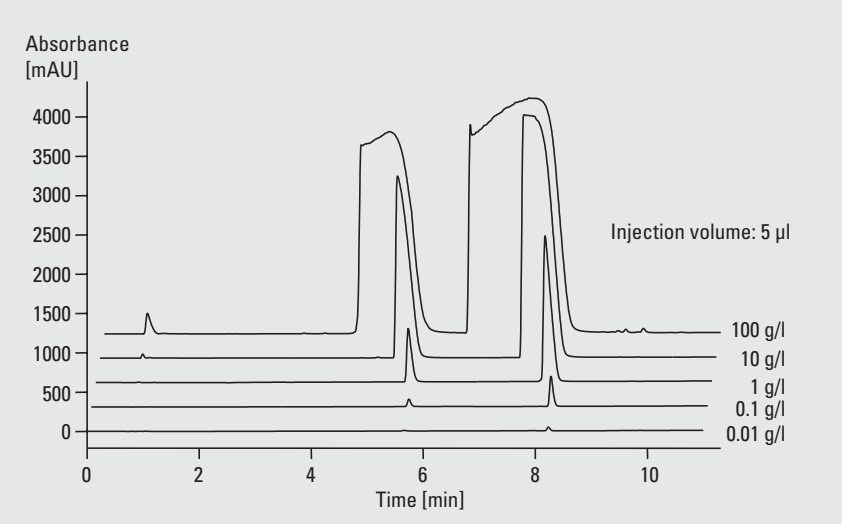

Figure 3b
Concentration overloading

Scale up to a semi-preparative column

The scale up calculation from the to analytical to the semi-preparative method is shown in figure 4.

Separation of a crude product sample using the Agilent 220 micro plate sampler

Since the crude product was very soluble in the liquid phase the sample amount of 2 mg per injection was achieved by increasing the sample concentration to 400 g/l. Therefore, the injected sample volume could be kept low at 5 µl. The chromatogram for the semi-preparative run is shown in figure 5.

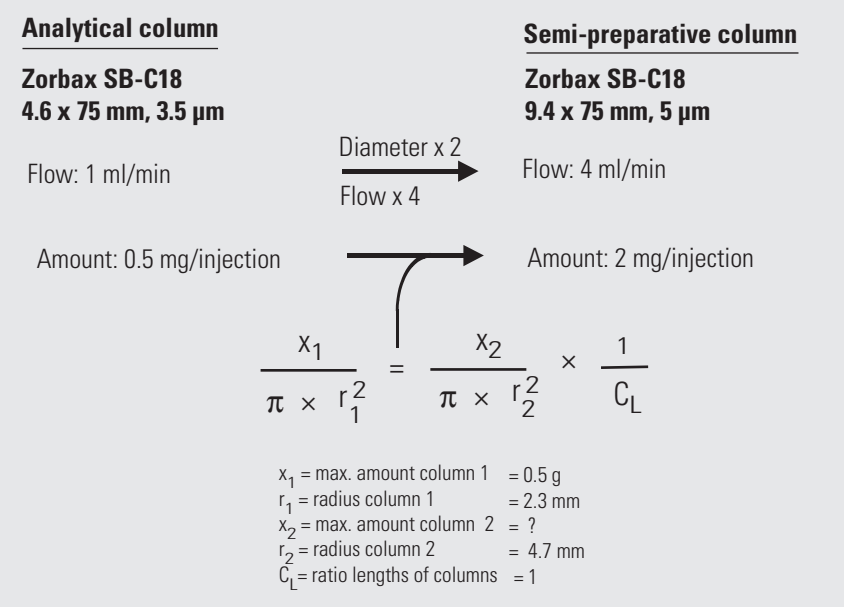
Analytical column
Zorbax SB-C18
4.6 x 75 mm, 3.5 µm
Flow: 1 ml/min
Amount: 0.5 mg/injection
Diameter x 2
Flow x 4
Semi-preparative column
Zorbax SB-C18
9.4 x 75 mm, 5 µm
Flow: 4 ml/min
Amount: 2 mg/injection
$\frac{x_1}{\pi \times r_1^2} = \frac{x_2}{\pi \times r_2^2} \times \frac{1}{C_L}$
x_1 = max. amount column 1 = 0.5 g
r_1 = radius column 1 = 2.3 mm
x_2 = max. amount column 2 = ?
r_2 = radius column 2 = 4.7 mm
C_L = ratio lengths of columns = 1

Figure 4
Scale up to semi-preparative method

Mobile Phases:	A = 0.025M KH_2PO_4 in water (pH=3), B = ACN
Gradient:	0 % B to 50 % B in 10 min 50 % B to 0 % B in 1 min
Flow	4 ml/min
Stop time:	11 min
Post time	5 min
Column:	Zorbax SB-C18, 4.6 x 75 mm, 5 µm
Column temp.:	40 °C
UV detector:	DAD 204 nm/16 (reference 360 nm/100)

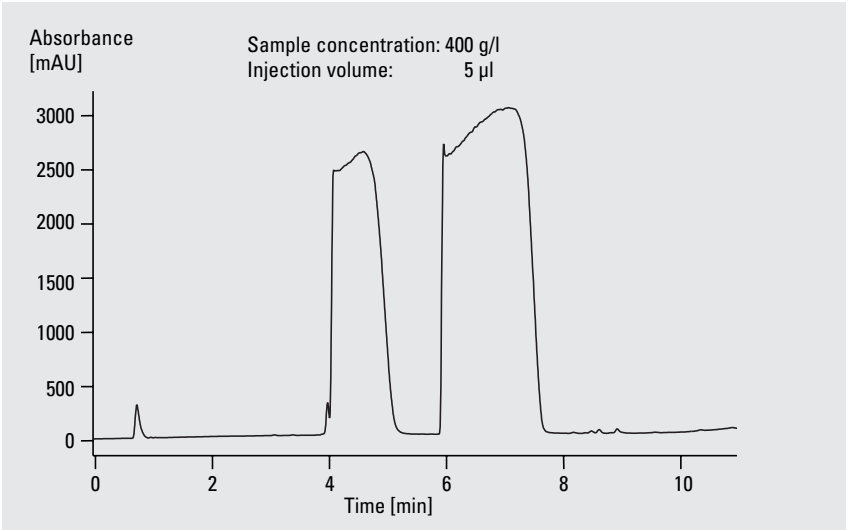

Figure 5
Semi-preparative separation of the crude product

Fractionation was done using time based fraction collection with the CC-Mode software. Eleven fractions per crude sample injection were taken and re-analyzed for purity. Since the main and side product had the same response factors at the measured wavelength the purity of the fractions could be determined directly from the chromatogram. Eight fractions contained the desired product and seven of them were pure enough to pool them. These combined fractions were also re-analyzed for purity (figure 6). The overall purity for the desired product was > 98 % (requirement: > 95 %) and the side product was < 1 % (requirement: < 2 %). Since the desired amount per injection of 2 mg was also achieved the semi-preparative separation of the crude synthesis product was successfully accomplished.

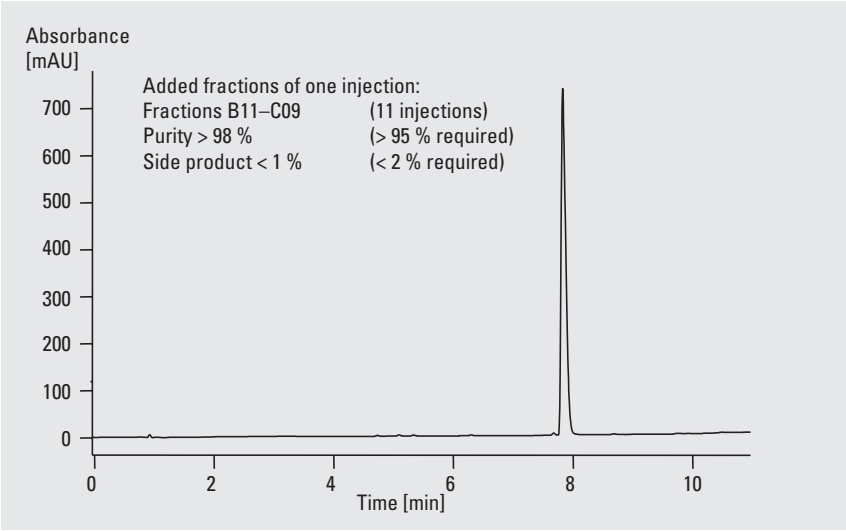

Figure 6
Re-analysis of the combined fractions

Conclusion

In this application note we showed the development of a semi-preparative method for purification of a crude synthesis product on the Agilent 220 MPS with the Agilent 1100 Series LC system. An analytical method was set up and optimized. The adsorption isotherm was measured to define the analytical and preparative range of the method. After volume and concentration overloading on the analytical column the method was scaled up to semi-preparative scale. The fractionation based on time windows and the re-analysis of the fractions were done using the Agilent 220 MPS controlled by the CC-Mode software.

In this application note we demonstrated that the Agilent 220 MPS together with the Agilent 1100 Series LC system can not only be used for analytical tasks, such as isolation and purification of natural products or analyses of combinatorial chemistry libraries, but also for semi-preparative purification using larger columns and higher flow rates.

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